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Cell specificity for the pulmonary metabolism of tobacco-specific nitrosamines in the Fischer rat

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The activity and distribution of the metabolic pathways of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), its major metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and the structurally related nitrosamine, *N'*-nitrosonornicotine (NNN) were examined in pulmonary cells from F344 rats in order to investigate the mechanisms by which NNK and NNAL, but not NNN, cause lung tumors. The tritium labeled nitrosamines were incubated with Clara cells, alveolar macrophages, alveolar type II cells, or small cells and metabolites were analyzed by HPLC. *O*^{6}-Methylguanine (*O*^{6}MG) formation was also quantified in the cells incubated with NNK. Clara cells metabolized all compounds more extensively than the other cell types. Total α-hydroxylation, carbonyl reduction to NNAL, and pyridine *N*-oxidation in cells incubated with NNK, as well as concentrations of *O*^{6}MG in DNA were higher in Clara cells than in other cell types. Carbonyl reduction of NNK predominated over the other metabolic pathways in all cell types. The high activity for α-hydroxylation of NNK in Clara cells is consistent with previous studies which proposed that the cell specificity for *O*^{6}MG formation and the accumulation of this adduct during low-dose exposure to NNK may stem from the presence of a high affinity pathway in Clara cells for NNK activation. Metabolism of NNAL by α-hydroxylation, and by reconversion to NNK followed by α-hydroxylation were observed. Total α-hydroxylation of NNAL was less extensive than α-hydroxylation of NNK. NNN was metabolized by both the 2'- and 5'-α-hydroxylation pathways. 2'-Hydroxylation of NNN produces the same DNA pyridyloxobutylating agent as does methyl hydroxylation of NNK. However, NNN is not a methylating agent and does not induce lung tumors in rats. Metabolism of NNN by 2'-hydroxylation was, depending on cell type, 41–85% as extensive as total α-hydroxylation of NNK, indicating that the rates of formation of the DNA pyridyloxobutylating agent were similar from NNN and NNK. The results of this study demonstrate that Clara cells have a high capacity to metabolically activate NNK, NNAL and NNN and provide further support for the hypothesis that DNA methylation of pulmonary cells is important in NNK carcinogenesis.

*Abbreviations: HPBS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered balanced salt solution; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK-*N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone; NNN, *N'*-nitrosonornicotine; *O*^{6}MG, *O*^{6}-methylguanine.

Introduction

The presence of many mutagens and carcinogens in tobacco smoke supports the causal relationship between cigarette smoking and lung cancer. One of the potent carcinogens in mainstream and sidestream smoke and unburned tobacco is the nicotine derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) (1–3). Chronic treatment of rats with NNK administered by s.c. injection or in the drinking water, result in the induction of adenocarcinoma and squamous cell carcinoma of the lung (2–4). Doses used in these studies are comparable to human exposure to NNK through cigarette smoking. Pulmonary tumors are also induced in hamsters and mice following either chronic or acute exposure to this carcinogen (2,3). Recent studies have indicated that NNK-induced pulmonary tumors from A/J mice may arise at least in part by activation of the K-*ras* oncogene (S.A.Belinsky, T.R.Devereaux, R.R.Maronpot, G.D.Stoner and M.W.Anderson, in preparation). Rodenhuis *et al.* (5) have also detected an activated K-*ras* oncogene in ~30% of human lung adenocarcinomas from smokers suggesting a strong association between activation of this proto-oncogene and smoking. These studies support our hypothesis that exposure to NNK may be an important contributing factor for the induction of lung cancer in smokers.

Metabolic activation of NNK is required to elicit its carcinogenicity. The metabolism of NNK (Figure 1) is known to occur by carbonyl reduction, *N*-oxidation, or by α-hydroxylation either at the methyl or *N*-methylene carbon (6). Carbonyl reduction produces 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a major metabolite of NNK and a potent pulmonary carcinogen (4,7). In contrast, *N*-oxidation results in formation of 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone (NNK-*N*-oxide, 1, Figure 1), a weaker carcinogen than NNK in the A/J mouse (7). α-Hydroxylation at the *N*-methylene carbon leads to methyl diazohydroxide 9, a methylating species which can react covalently to modify DNA by forming methylated adducts. α-Hydroxylation of NNK at the *N*-methyl carbon can yield the pyridyloxobutyl diazohydroxide 7. This intermediate reacts with DNA to form an adduct of unknown structure (12). The role of this adduct in neoplasia by NNK has not been determined. However, treatment of rats with *N'*-nitrosonornicotine (NNN), a nitrosamine whose activation also leads to the formation of this adduct, does not induce pulmonary tumors (13). Prior to this study, there was no information available concerning the cellular distribution of NNK activation and detoxification pathways in rat lung.

The promutagenic adduct O^6-methylguanine (*O*^{6}MG) has been detected in lungs of rats treated with doses of NNK ranging from 0.1 to 100 mg/kg (8). Accumulation of this adduct has been observed in lung after treatment with either high or low doses of NNK. However, the quantitation of DNA methylation in whole lung may be misleading since there are at least 40 different pulmonary cell types (9). Measurement of *O*^{6}MG in specific pulmonary cell populations following exposure to NNK revealed a cell specificity for methylation of DNA. Concentrations of

DNA isolation and determination of DNA adducts

After incubation of lung cells with [5-^{3}H]NNK or [5-^{3}H]NNN, DNA was isolated from cell pellets by digestion with pronase in 1% SDS, followed by phenol–chloroform extraction and ethanol precipitation (23). Samples were incubated with RNase T1 (100 units; Sigma Chemical Co., St Louis, MO) and RNase A (300 units, Sigma) for 3 h at 37°C and DNA was recovered by ethanol precipitation. This procedure removed all RNA contamination as detected by electrophoresis of DNA on agarose gels.

The measurement of the both O^6MG and keto alcohol 12 in cells incubated with NNK, and keto alcohol 12 in cells incubated with NNN, was accomplished following neutral thermal or strong acid hydrolysis of DNA as described (10,12). The supernatants were analyzed for keto alcohol 12 by reverse-phase HPLC, system 1. Limits of detection were ~1 pmol/μg DNA for Clara cells and macrophages and 0.3 pmol/μg DNA for alveolar type II and small cells. The DNA that was precipitated with ethanol after neutral thermal hydrolysis was lyophilized and resuspended in Tris buffer (1 mM, pH 7.4). DNA was then enzymatically digested to deoxyribonucleosides using DNase I, alkaline phosphatase and snake venom phosphodiesterase as previously described (24). Fifty μl were removed from each sample and subjected to reverse-phase HPLC with integration of UV absorbing peaks to determine the concentration of normal nucleotides. Digested DNA samples (0.2–0.7 mg) were chromatographed using reverse-phase HPLC and fractions collected, dried under reduced pressure and then analyzed by radioimmune assay to determine the amount of O^6MG present (24). The antibody to O^6-methyldeoxyguanosine was a gift from Dr James Swenberg. Limits of detection were 0.35, 0.1, 0.1 and 0.5 pmol/μmol for macrophages, small cells, type II cells and Clara cells, respectively.

Results

Metabolism of [5-^{3}H]NNK by pulmonary cells

In order to optimize the conditions to study the metabolism of NNK, the time course for metabolite formation was determined in all four cell types during incubation for 0–60 min with 50 μM [5-^{3}H]NNK at a sp. act. of 8.2 mCi/mmol. The major metabolite detected in all cell types was NNAL. Its formation was approximately linear throughout the incubation period in Clara cells, alveolar macrophages and type II cells (Figure 2). Very little metabolism of NNK or NNAL was observed in small cells. Rates of formation of NNAL were greatest in Clara cells, followed by macrophages, type II cells and small cells. Keto acid 13 (Figure 1) was detected in Clara cells after 30, 45 and 60 min of incubation, reaching a maximum concentration of 30 pmol/10^6 cells (data not shown). Formation of keto alcohol 12 at a concentration of 30 pmol/10^6 cells was also observed in Clara cells after 60 min of incubation with [5-^{3}H]-NNK.

The sensitivity of the detection of NNAL, keto alcohol 12 and keto acid 13 was increased by incubation of lung cells with 150 μM [5-^{3}H]NNK at a sp. act. which was 8-fold greater than that used in the previous experiment (64 mCi/mmol). Under these conditions, NNK-*N*-oxide 1 was observed in Clara cells and type II cells (Table I). Keto acid 13 was detected in all four cell types and rates of formation appeared greatest in Clara cells, followed by alveolar macrophages, alveolar type II cells and small cells. The amounts of keto alcohol 12 were similar to those of keto acid 13 in Clara cells and type II cells (Table I). In contrast, this metabolite was not detected in either alveolar macrophages or small cells.

The metabolites formed from incubation with [5-^{3}H]NNK were identified and quantitated by analyzing the media by reverse-phase HPLC using system 1 (Figure 3). The identity of keto acid 13 was confirmed by collecting the peak observed at 24 min (Figure 3) and analyzing this aliquot by reverse-phase HPLC using system 2. This metabolite, with a retention time of 38 min, was present in all cell types using system 2. Keto aldehyde 8 and lactol 10 elute with the same retention time as keto alcohol 12 using system 1, but can be resolved by normal phase HPLC. This peak was collected and analyzed by normal phase HPLC in a separate experiment in which the sp. act. of NNK was increased to 818 mCi/mmol to facilitate detection of these products. The presence of keto alcohol 12 was confirmed, but neither keto aldehyde 8 nor lactol 10 was detected in this analysis. The increase in sensitivity gained from this experiment enabled the detection of NNK-*N*-oxide 1 in small cells (4.2 pmol/10^6 cells) and keto acid 12 in small cells and alveolar macrophages (3.8 and 14.5 pmol/10^6 cells, respectively).

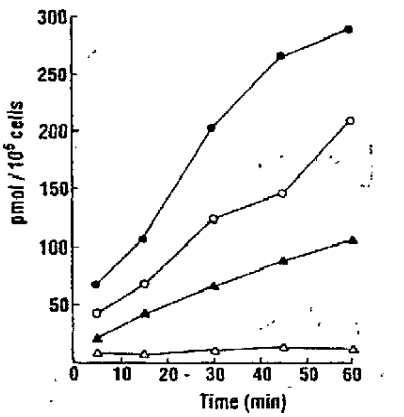


Fig. 2. Formation of NNAL in lung cell types incubated for 0–60 min with [5-^{3}H]NNK. ●—●, Clara; ○—○, alveolar macrophage; ▲—▲, type II; △—△, alveolar small.

Metabolism of [5-^{3}H]NNAL

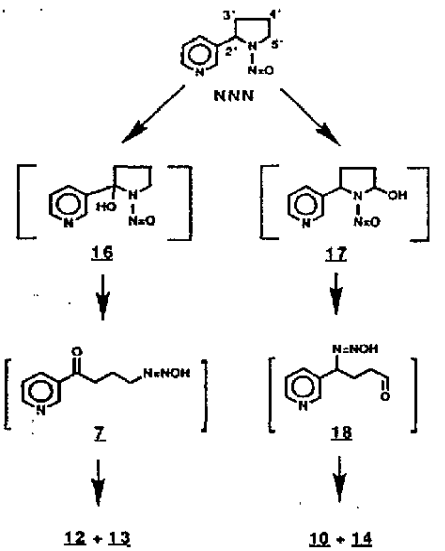
Two metabolites, hydroxy acid 14 and keto acid 13 were detected in media following incubation of the four pulmonary cell types with [5-^{3}H]NNAL (Table I). NNK was not detected in these studies. The identity of hydroxy acid 14 was verified by collecting it from HPLC system 1 and analyzing the peak in system 3. Rates of metabolism of NNAL appeared similar in alveolar macrophages, type II cells and small cells. Metabolism of NNAL was ~3- to 4-times greater in Clara cells than in the other pulmonary cell types (Table 1).

Metabolism of [5-^{3}H]NNN

Three metabolites, hydroxy acid 14, keto alcohol 12 and keto acid 13 were detected in media from pulmonary cells incubated with [5-^{3}H]NNN (Table I, Figure 4). Metabolism of NNN was greatest in Clara cells followed by macrophages, type II cells and small cells. The distribution of metabolites varied significantly among cell types. The ratio of hydroxy acid 14, formed by 5′-hydroxylation of NNN, to keto alcohol 12 and keto acid 13, formed by 2′-hydroxylation (Figure 4), was similar in Clara cells and type II cells (Table I). In contrast, 2′-hydroxylation predominated in macrophages and small cells.

DNA adduct formation in pulmonary cells

Methylation and pyridyloxobutylation of DNA were quantitated in pulmonary cell types by measurement of O^6MG and keto alcohol 12. The concentrations of O^6MG (pmol/μmol deoxyguanosine) detected in the four cell types were: Clara cells, 2.35; alveolar macrophages, <0.35; alveolar type II, 0.28; small cells, 0.53. The release of keto alcohol 12 was not observed (limits of detection, 1 pmol/μmol deoxyguanosine) from DNA of lung cells which had been incubated with either NNK or NNN.



. 4. Metabolism of NNN by α-hydroxylation. The structures of keto alcohol 12, keto acid 13, lactol 10, and hydroxy acid 14 are shown in Figure 1.

toward the reductive pathway over the α-hydroxylation pathways. In addition, while reduction of NNK to NNAL was readily detected in all cell types, *N*-oxidation was not observed in macrophages and was detected in small cells only after incubation with NNK of high specific activity. Thus, enzyme activities for carbonyl reduction and *N*-oxidation of NNK appear to be distributed heterogeneously within the rat lung.

The potency of NNK as a carcinogen may be augmented by its rapid conversion to NNAL (25). The half life of NNAL, which is also a pulmonary carcinogen, is ~5 h compared to 30 min for NNK in the Fischer rat (25). Thus, in animals treated with NNK, the lungs will also be exposed to NNAL. Therefore, the metabolic pathways of NNK and NNAL were compared in lung cells. The results indicate that NNAL was metabolized directly or following reconversion to NNK. Hydroxy acid 14, formed by α-hydroxylation of NNAL, was the predominant metabolite detected in all cell types. α-Hydroxylation of NNAL to hydroxy id 14 also has been observed in cultured human bronchus and peripheral lung incubated with NNK (26). Keto acid 13, which is most likely formed following metabolism of NNAL to NNK, was also detected in all cell types. Total α-hydroxylation of NNAL was less extensive than α-hydroxylation of NNK. This is consistent with lower levels of methylated guanines present in lung DNA of rats treated with NNAL compared to NNK (27).

Comparative bioassays of NNK and NNN in the rat have clearly shown that NNK is a strong lung carcinogen whereas NNN is not (13,28). For example, after injection of a total dose of 1 mmol/kg, 23 of 27 F344 rats treated with NNK had lung tumors, whereas the incidence of lung tumors in the NNN-treated group was 0 (13). Previous studies have shown that NNK and NNN can both be metabolized by a common pathway leading to diazohydroxide 7 (6,18). However, only NNK is metabolized to methyl diazohydroxide 9, a DNA methylating agent (29). This suggested that methylation of DNA might be important in the induction of lung tumors by NNK in the rat (8,28). However, it was also possible that NNK was far more extensively metabolized to diazohydroxide 7, than was NNN. Comparison of the metabolism of NNK and NNN demonstrated that, depending on the cell type, the extent of 2′-hydroxylation of NNN ranged from 41 to 85% of the extent of total α-hydroxylation of NNK. These results indicate that levels of diazohydroxide 7 formed from NNK in lung cells do not greatly exceed the levels formed from NNN, and support the hypothesis that DNA methylation *via* methyl diazohydroxide 9 is an important component of NNK's ability to induce lung tumors.

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